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PRINCIPAL INVESTIGATOR: Yan Dong

CONTRACTING ORGANIZATION: Tulane University Health Science Center
New Orleans, LA 70112-2699

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Yan Dong

E-Mail: ydong@tulane.edu

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Tulane University
1430 Tulane Avenue
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14. ABSTRACT

Constitutively-active androgen receptor splice variants (AR-Vs) that lack the ligand-binding domain have been implicated to play an important role in mediating castration-resistant progression of prostate cancer. AR-Vs have been shown to regulate the expression of both canonical AR targets and a unique set of targets enriched for cell-cycle function. However, little is known about how regulation of gene expression by AR-Vs is achieved. We are the first to show that AR-Vs not only homodimerize and heterodimerize with each other but also heterodimerize with the full-length AR (AR-FL). Significantly, dimerization is required for the *trans*-activating activity of AR-Vs. To date, the ability of AR-Vs to regulate gene expression has been attributed largely to their AR-FL-independent activity. Based on our finding and the fact that AR-Vs are often co-expressed with AR-FL in biological context, we believe that the ability of AR-V to heterodimerize with and activate AR-FL in an androgen-independent manner could be as important, if not more important, than its AR-FL-independent activity to castration resistance. The research therefore represents a key step in delineating the mechanism of activation of AR-Vs, which is vital for developing effective means to suppress AR-V signaling for more effective treatment of prostate cancer.

15. SUBJECT TERMS
Androgen receptor, splice variant, dimerization, prostate cancer, castration resistance

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INTRODUCTION:

A major challenge in treatment of advanced prostate cancer is the development of castration-resistant prostate cancer after androgen deprivation therapy (1). Despite the depletion of circulating androgens, androgen receptor (AR) signaling remains active in the tumors, constituting a major mechanism of castration-resistant progression (1). Recently, accumulating evidence points to the important contribution of constitutively-active, ligand-binding-domain-truncated AR splice variants (AR-Vs) to castration resistance (2-6). AR-Vs have been shown to regulate the expression of both canonical AR targets and a unique set of targets enriched for cell-cycle function (3,5,7). The full-length AR (AR-FL) needs to dimerize to regulate target-gene expression (8), but little is known about AR-V dimerization. The purpose of this study is to define the dimeric nature of AR-Vs. The bimolecular fluorescence complementation (BiFC) assay and the bioluminescence resonance energy transfer (BRET) assay were used to address this issue.

BODY:

Task 1. To generate BRET- and BiFC-fusion constructs of AR^{v567es}, AR-V7, and AR-FL and to functionally validate the fusion proteins.

BiFC-fusion constructs: AR^{v567es} and AR-V7 are two major AR-Vs expressed in castration-resistant prostate cancer specimens, and high expression has been correlated with short survival of the patients (9). To generate different BiFC-fusion constructs of AR-FL, AR^{v567es}, and AR-V7, we PCR amplified human AR-FL, AR^{v567es}, and AR-V7 cDNAs from their respective expression constructs, and cloned the PCR amplicons separately into a TA-cloning vector. Fusion constructs of AR-FL, AR^{v567es}, and AR-V7 with either the N- or C-terminal fragment of the Venus fluorescent protein (VN- or VC-VFP) were then generated by subcloning the cDNAs from the TA-vectors into the SalI and XhoI sites of the pBiFC-VN155 and pBiFC-VC155 vectors (kindly provided by Dr. Chang-Deng Hu at Purdue University). To delineate the dimerization interface, we generated mutant BiFC-AR-V constructs with mutation at the FxxLF motif (F23,27A/L26A) and/or D-box (A596T/S597T), which mediate AR-FL homodimerization through N/C-terminal interaction or DNA-binding domain (DBD)/DBD interaction, respectively (8). The mutant constructs were generated by site-directed mutagenesis by using the Q5 site-Directed Mutagenesis Kit (New England BioLabs). Each fusion construct was transiently transfected into the AR-null PC-3 cell line, and the expression of the fusion proteins confirmed by Western blot analyses (Fig. 1). The ability of the wild-type fusion proteins to *trans*-activate was validated by luciferase assay with the co-transfection of an androgen-responsive element (ARE)-luciferase reporter plasmid containing three ARE regions ligated in tandem to the luciferase reporter, ARR3-luc (Fig. 2).

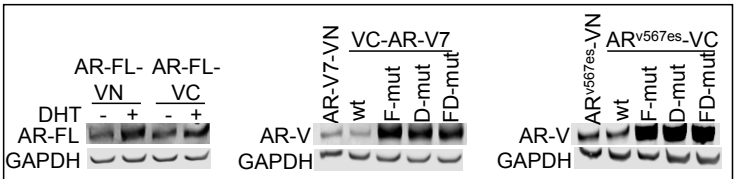

Fig. 1. Expression of various BiFC-fusion proteins. Individual fusion construct was transfected into PC-3 cells cultured under androgen-deprived condition. Dihydrotestosterone (DHT), at 1 nM, was added to the cells at 24 hr after transfection. After an additional 24 hr of incubation, cell lysates were collected and subjected to Western blot analyses with an antibody against the N-terminus of AR (PG-21). wt, wild-type; F-mut, FxxLF-motif mutant; D-mut, D-box mutant; FD-mut, FxxLF-motif and D-Box double mutant.

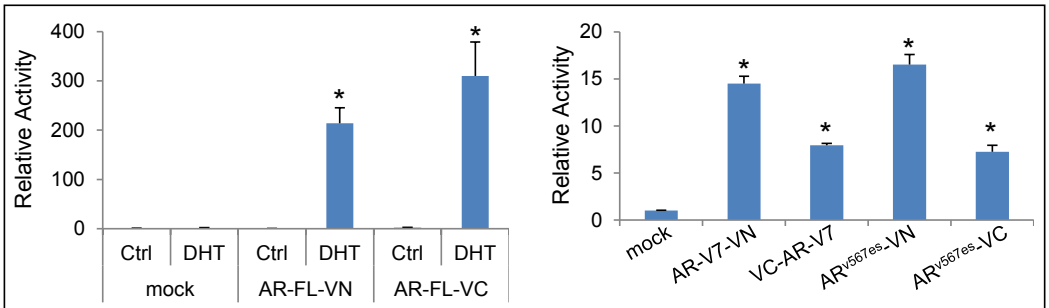

Fig. 2. *Trans*-activating activity of various BiFC-fusion proteins. Individual fusion construct was co-transfected into PC-3 cells with ARR3-luc, a luciferase construct for measuring AR *trans*-activating activity. Cells were cultured under androgen-deprived condition, and 1 nM DHT was added 24 hr after transfection. After an additional 24 hr of incubation, cells were harvested for luciferase assay. Data are presented as relative to mock control. *, $P < 0.05$ from mock control.

BRET-fusion constructs: Different BRET-fusion constructs of AR-FL, AR^{v567es}, and AR-V7 were generated by subcloning the AR-FL, AR^{v567es}, and AR-V7 cDNA from the respective TA-vectors into the BamHI and XbaI sites of the RLuc8.6 luciferase construct and the TurboFP635 construct (10) (kindly provided by Dr. Sanjiv Gambhir at Stanford University). Each fusion construct was then transiently transfected into PC-3 cells, and the expression of the fusion proteins confirmed by Western blot analyses (Fig. 3). The ability of the fusion proteins to regulate target gene expression was validated by qRT-PCR analysis of prostate-specific antigen (PSA) levels (Fig. 4).

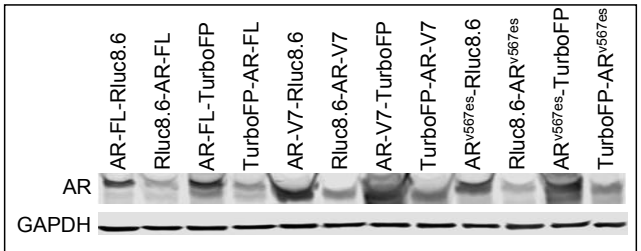

Fig. 3. Expression of various BRET-fusion proteins. Individual fusion construct was transfected into PC-3 cells cultured under androgen-deprived condition. Cell lysates were collected at 48 hr after transfection and subjected to Western blot analyses with an antibody against the N-terminus of AR (PG-21).

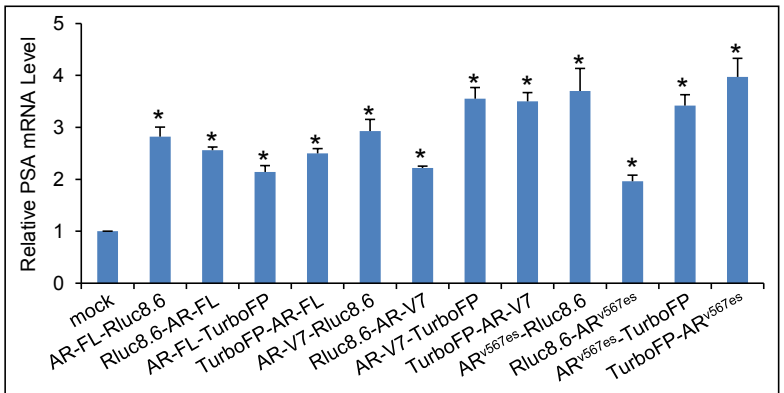

Fig. 4. Ability of various BRET-fusion proteins to regulate gene expression. Individual fusion construct was transfected into LNCaP cells. Cells were cultured under androgen-deprived condition, and harvested at 72 hr after transfection. The levels of PSA mRNA were assessed by qRT-PCR. The data are presented as relative to mock control. *, $P < 0.05$ from mock control.

Task 2. To delineate dimer formation among AR^{v567es}, AR-V7, and AR-FL, and response to androgen and anti-androgen.

BiFC detection of AR-V/AR-FL heterodimerization: For BiFC detection of AR-V/AR-FL dimerization, we co-transfected the AR-V- and AR-FL BiFC fusion proteins into PC-3 cells. The principle of the BiFC assay is that if two ARs dimerize, the interaction can re-generate the Venus fluorescent protein to emit fluorescent signal (11). We found that both AR-V7 and AR^{v567es} dimerize with AR-FL, and that the dimerization does not require androgen (Fig. 5). Only mutating both the FxxLF motif and the D-Box abolished AR-FL/AR-V dimerization, indicating that both N/C and DBD/DBD interactions mediate the dimerization. Mutating one motif did not lead to significant change of BiFC signal is possibly due to compensation of the loss of one mode of interaction by the other. Similar result was obtained in DU145 cells (Fig. 6). Intriguingly, although AR-FL/AR^{v567es} dimerization was observed in both the cytoplasm and nucleus, AR-FL/AR-V7 dimerization was detected primarily in the nucleus. On the other hand, we found that, similar to AR^{v567es} (5), AR-V7 induces AR-FL nuclear entry (Fig. 7), indicating their initial interaction in the cytoplasm. Thus, the inability to detect cytoplasmic dimerization is likely because VFP re-generation from the VN and VC fragments is slower than AR-V7/AR-FL nuclear translocation. Of note, immunofluorescence assay was used to make sure the protein fusion did not alter the subcellular localizations of the AR isoform (Fig. 8). Moreover, we showed that pretreatment of cells with dihydrotestosterone (DHT) attenuated AR-V7/AR-FL heterodimerization, and this effect was blocked by the antiandrogen MDV3100 (Fig. 9), indicating potential competition of AR-V with AR-FL for dimerizing with AR-FL.

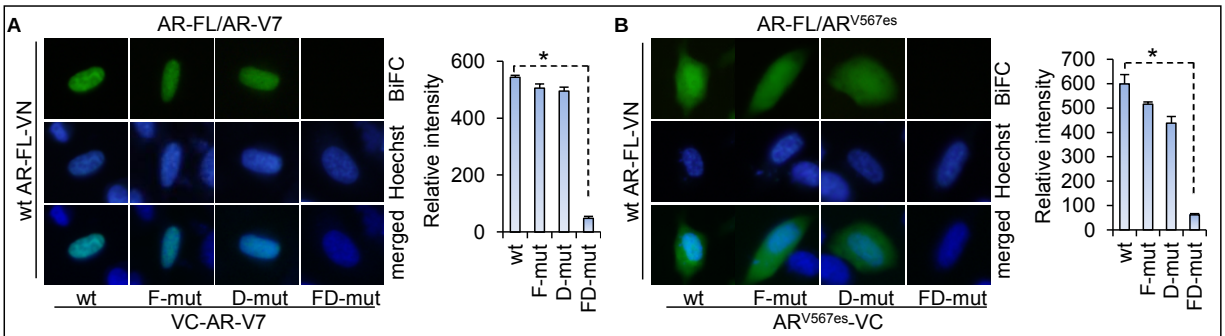

Fig. 5. AR-V heterodimerizes with AR-FL through both N/C and DBD/DBD interactions. Dimerization was detected by BiFC assay in PC-3 cells under androgen-deprived condition. Right panels, quantitation of BiFC signals by flow cytometry. wt, wild-type; F-mut, FxxLF-motif mutant; D-mut, D-box mutant; FD-mut, FxxLF-motif and D-Box double mutant. *, $P < 0.01$.

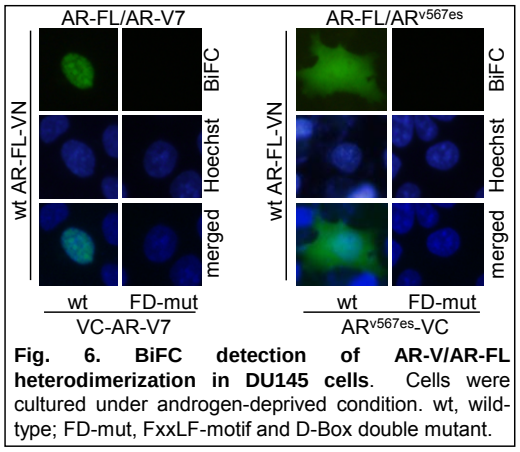

Fig. 6. BiFC detection of AR-V/AR-FL heterodimerization in DU145 cells. Cells were cultured under androgen-deprived condition. wt, wild-type; FD-mut, FxxLF-motif and D-Box double mutant.

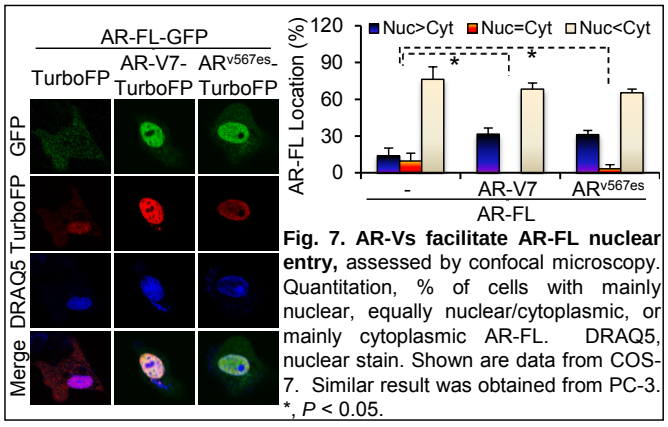

Fig. 7. AR-Vs facilitate AR-FL nuclear entry, assessed by confocal microscopy. Quantitation, % of cells with mainly nuclear, equally nuclear/cytoplasmic, or mainly cytoplasmic AR-FL. DRAQ5, nuclear stain. Shown are data from COS-7. Similar result was obtained from PC-3. *, $P < 0.05$.

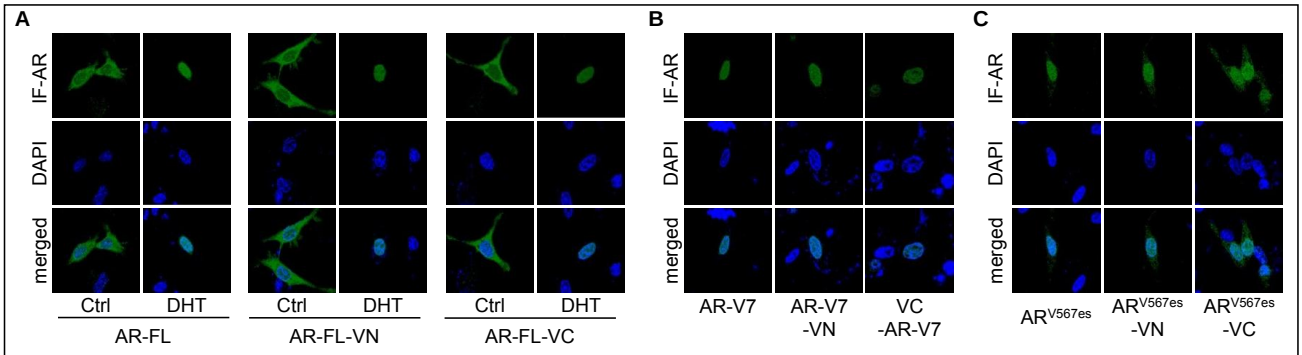

Fig. 8. Protein fusion does not change subcellular localization of AR-FL or AR-V. AR-FL or AR-V expression construct or AR-FL or AR-V BiFC construct was transfected into PC-3 cells, and AR subcellular localization was detected by immunofluorescence staining at 48 hr after transfection. Cells were cultured under androgen-deprived condition, and 1 nM DHT was added 24 hr after transfection.

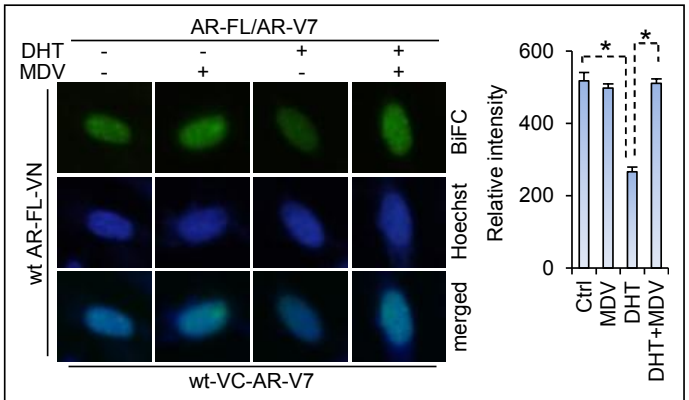

Fig. 9. Pretreatment with DHT attenuates AR-V/AR-FL heterodimerization. AR-FL-VN and VC-AR-V7 constructs were co-transfected into PC-3 cells. Cells were treated with 1 nM DHT with or without 10 μM MDV3100 (MDV) right after transfection, and BiFC signal was assessed at 48 hr after transfection. Right panel, quantitation of BiFC signals by flow cytometry. *, $P < 0.01$.

<u>BiFC detection of AR-V/AR-V dimerization</u>: We further found that, like liganded AR-FL (Fig. 10A), both AR-Vs can also form a homodimer (Fig. 10B and 10C; Fig. 11). Moreover, AR-V7 and AR^{v567es} can also heterodimerize (Fig. 10D). Mutating the D-box, but not the FxxLF motif, abolished the interactions, indicating that AR-Vs homodimerize and heterodimerize with each other through DBD/DBD interaction. We also generated pBiFC-CN155 and pBiFC-CC155 fusion constructs of AR-V7 and AR^{v567es} for the purpose of confirming the competition of AR-V with AR-FL for dimerizing with AR-FL by using the BiFC protocol of simultaneous visualization of multiple protein interactions. Unfortunately, we still have technical difficulty of getting the assay to work. We therefore decide to use BRET assay in the future to further demonstrate the competition.

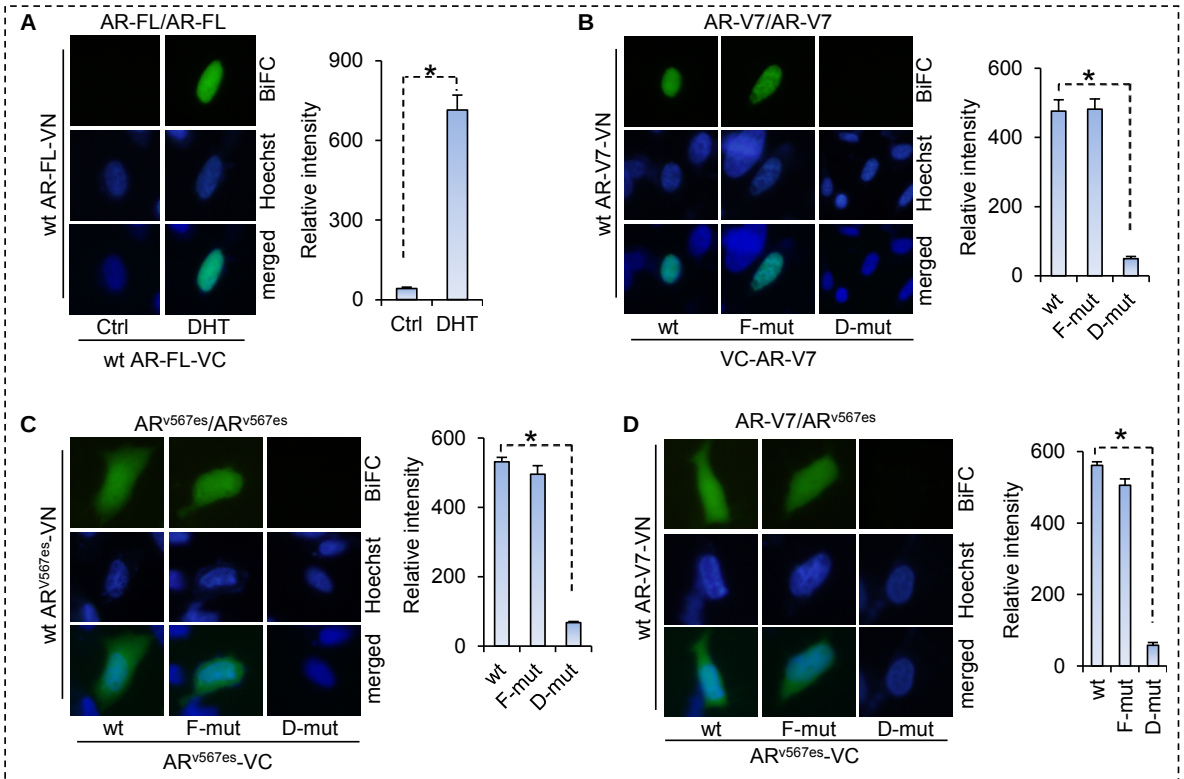

Fig. 10. AR-V and AR-V dimerize through DBD/DBD interaction. BiFC detection of AR-FL homodimerization (A), AR-V7 homodimerization (B), AR^{v567es} homodimerization (C), and AR-V7/AR^{v567es} heterodimerization in PC-3 cells under androgen-deprived condition unless specified. DHT, 1 nM for 24 hr. Right panels, quantitation of BiFC signals by flow cytometry. . wt, wild-type; F-mut, FxxLF-motif mutant; D-mut, D-box mutant. *, $P < 0.01$.

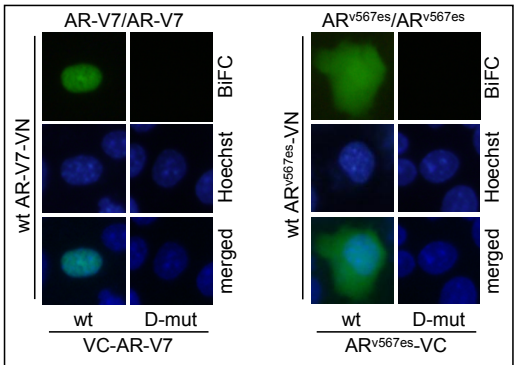

Fig. 11. BiFC detection of AR-V homodimerization in DU145 cells. Cells were cultured under androgen-deprived condition. wt, wild-type; D-mut, D-Box mutant.

<u>BRET confirmation of AR-V/AR-FL and AR-V/AR-V dimerization</u>: BRET is based on energy transfer between a luciferase energy donor and a fluorescent protein energy acceptor when the donor and acceptor are brought into close proximity by their fused proteins (10). Different combinations of the fusion proteins were tested in PC-3 cells. Fig. 12 is the saturation curve for different combinations, showing BRET ratios increase hyperbolically and rapidly saturate, indicating specific protein-protein interaction (12). Thus, the BRET data confirmed the BiFC results, showing the ability of AR-V to homodimerize and to heterodimerize with AR-FL and another AR-V. We are in the process of characterizing the effect of androgen and anti-androgen MDV3100 on AR-V/AR-FL dimerization by the BRET assay.

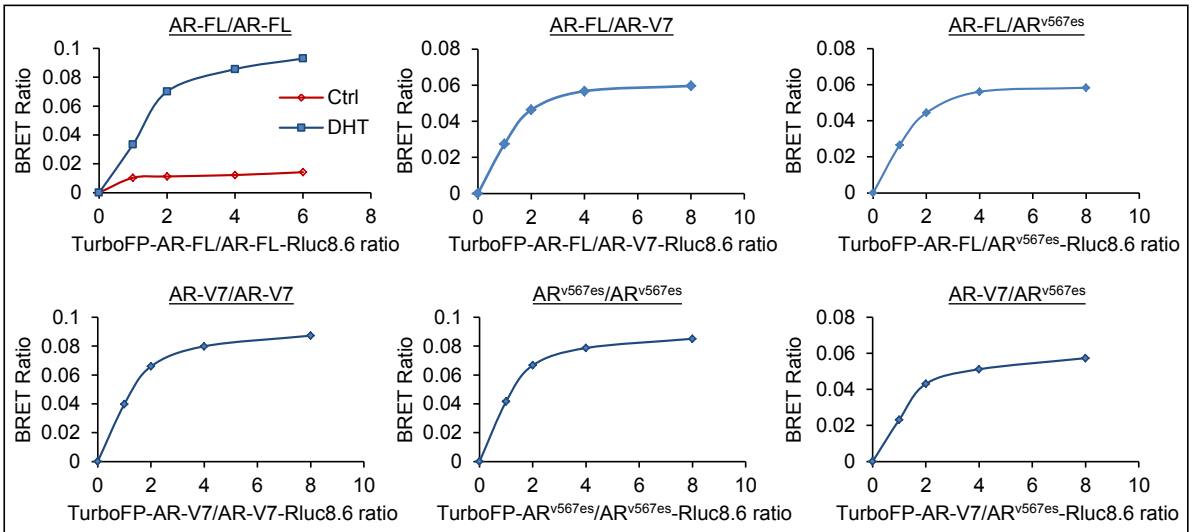

Fig. 12. BRET saturation curve. Indicated TurboFP and Rluc8.6 fusion constructs were co-transfected into PC-3 cells at different ratios, and BRET signal measured after addition of the coelenterazine substrate. Cells were cultured under androgen-deprived condition unless specified. DHT, 1 nM for 24 hr.

Dimerization is required for transactivation: We co-transfected the wild-type or the dimerization mutants of AR-V with the ARR3-luc construct into PC-3 cells, and found that the dimerization mutants completely lost the ability to *trans*-activate (Fig. 13), indicating an absolute requirement of dimerization for AR-V transactivation.

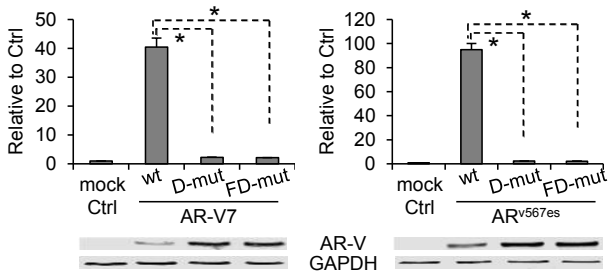

Fig. 13. Dimerization mutants of AR-Vs lose ability to *trans*-activate. Wild-type or dimerization mutant of AR-V was co-transfected into PC-3 cells with ARR3-luc, a luciferase construct for measuring AR *trans*-activating activity. Western blotting confirmed AR-V expression.

KEY RESEARCH ACCOMPLISHMENTS:

- We showed that AR-V can form a heterodimer with AR-FL through N/C and DBD/DBD interactions, and the dimerization does not require androgen.
- We showed that AR-V can homodimerize or dimerize with another AR-V through DBD/DBD interaction, and the dimerization does not require androgen.
- We identified the dimerization interface.
- We showed that, similar to AR^{v567es}, AR-V7 can induce AR-FL nuclear translocation.
- We showed potential competition of AR-V with AR-FL for dimerizing with AR-FL.
- We showed that dimerization is required for AR-V transactivation.

REPORTABLE OUTCOMES:

- Meeting abstract:

  Bo Cao, Yanfeng Qi, and **Yan Dong**. ROLE OF ANDROGEN RECEPTOR SPLICE VARIANTS IN DETERMINING THE SENSITIVITY OF PROSTATE CANCER TO THE NEXT−GENERATION

ANTIANDROGEN MDV3100. In 2012 Society for Basic Urology Research Fall Symposium Program Book, 2012 Nov 15-18; Miami, FL. Abstract nr 123.

- Manuscript submitted:

  Bo Cao, Yanfeng Qi, Guanyi Zhang, Duo Xu, Xavier Alvarez, Zhiyong Guo, Xueqi Fu, Stephen Plymate, Oliver Sartor, Haitao Zhang, and **Yan Dong**. (2013) Androgen Receptor Splice Variants Coalescing Androgen Receptor in Mediating Resistance to Androgen-Directed Therapy.

- Funding applied for based on work supported by this award:

  DOD Idea Development Award application entitled "Inherent mechanism of resistance to next-generation androgen deprivation therapy", submitted on 10/2/13

- List of personnel receiving pay from the research effort:

  Yan Dong (PI)

  Yanfeng Qi (postdoctoral fellow)

CONCLUSION:

AR-Vs have been shown to regulate the expression of both canonical AR targets and a unique set of targets enriched for cell-cycle function (3,5,7). However, little is known about how regulation of gene expression by AR-Vs is achieved. We are the first to show that AR-Vs not only homodimerize and heterodimerize with each other but also heterodimerize with AR-FL. Significantly, dimerization is required for the *trans*-activating activity of AR-Vs. To date, the ability of AR-Vs to regulate gene expression has been attributed largely to their AR-FL-independent activity. Based on our finding and the fact that AR-Vs are often co-expressed with AR-FL in biological context, we believe that the ability of AR-V to heterodimerize with and activate AR-FL in an androgen-independent manner could be as important, if not more important, than its AR-FL-independent activity to castration resistance. The research therefore represents a key step in delineating the mechanism of activation of AR-Vs, which is vital for developing effective means to suppress AR-V signaling for more effective treatment of prostate cancer.

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